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# Cardiac Hypertrophy After Transplantation Is Associated With Persistent Expression of Tumor Necrosis Factor- $\alpha$

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**Background**—The mechanisms that contribute to cardiac allograft hypertrophy are not known; however, the rapid progression and severity of hypertrophy suggest that nonhemodynamic factors may play a contributory role. Tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) is a cytokine produced in cardiac allografts and capable of producing hypertrophy and fibrosis; therefore, we suggest that TNF- $\alpha$  may play a contributory role. Accordingly, the aims of our study were to define the role of systemic hypertension in the development of hypertrophy, characterize the histological determinants of hypertrophy, and characterize the expression of myocardial TNF- $\alpha$  after heart transplantation.

**Methods and Results**—To separate the effect of hypertension from immune injury in the development of cardiac allograft hypertrophy, we measured the gain in left ventricular mass by 2D echocardiography in heart transplant recipients and lung transplant recipients who developed similar rates of systemic hypertension. The gain in left ventricular mass was 73% in heart transplant recipients and 7% in lung transplant recipients ( $P < 0.0001$ ). By comparing myocardial samples obtained during the first week after transplant and at 1 year, we found that there was a significant increase in total collagen content ( $P < 0.0001$ ), collagen I ( $P < 0.0001$ ), collagen III ( $P < 0.0001$ ), and myocyte size ( $P < 0.0001$ ). These changes were associated with persistent myocardial TNF- $\alpha$  expression.

**Conclusions**—We suggest that the contribution of hypertension to cardiac allograft hypertrophy is minimal and that persistent intracardiac expression of TNF- $\alpha$  may contribute to the development of cardiac allograft hypertrophy. (*Circulation*. 2001;104:676-681.)

**Key Words:** growth substances ■ transplantation ■ hypertrophy

The mechanisms responsible for the development of cardiac allograft hypertrophy are not known; however, the consequences of hypertrophy after transplantation are significant and potentially provide an explanation for the appearance of diastolic dysfunction and exercise intolerance, as well as the limited life span of cardiac allografts.<sup>1,2</sup> There are 2 potential mediators of hypertrophy after cardiac transplantation: first, the signals provided by systemic hypertension that frequently occur after transplantation, and second, immune injury or cytokines produced in response to the allograft that are capable of producing hypertrophy and or cardiomyopathy.

Tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) is a cytokine produced in the myocardium under pressure or volume overload but not present in normal hearts.<sup>3,4</sup> Under short-term experimental conditions, TNF- $\alpha$  induces negative inotropic effects,<sup>5,6</sup> but when chronically overexpressed in the myocardium, TNF- $\alpha$  leads to hypertrophy, cardiac enlargement, and death.<sup>7,8</sup> With regard to

the presence of TNF- $\alpha$  in cardiac allografts, preliminary studies from our laboratory, as well as from others, indicated that intracardiac TNF- $\alpha$  was elevated in heart transplant recipients in the absence of histological evidence of rejection.<sup>9-11</sup> Considering that TNF- $\alpha$  was present in cardiac allografts and is capable of inducing hypertrophy and fibrosis, we hypothesized that if TNF- $\alpha$  was persistently expressed, it might play a role in the development of cardiac allograft hypertrophy. Accordingly, the objectives of the present study were to define the effect of systemic hypertension on the development of hypertrophy, the histological characteristics of cardiac allograft hypertrophy, and the course of expression of TNF- $\alpha$  in heart transplant recipients.

## Methods

### Patient Characteristics

The studies were conducted after written informed consent was obtained from each patient and with approval of the Institutional

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**Demographics**

	Heart TX	Lung TX	P
n	10	18	...
Male	8	6	...
Female	2	12	...
Mean age, y	51	50	NS
Mean BP,* mm Hg	101	100	NS
CYA dose, mg	238 (n=8)	218 (n=10)	NS
CYA level, ng/mL	238 (n=8)	312 (n=7)	NS
Prednisone, mg	5 (n=9)	10 (n=14)	0.001
Azathioprine, mg	83 (n=3)	63 (n=4)	NS
MMF, mg	1000 (n=3)	1500 (n=2)	NS
FK506 dose, mg	6 (n=2)	6 (n=5)	NS
FK506 level, ng/ml	6 (n=1)	11 (n=4)	NA
Anti-hypertensive drug†	7/10	10/18	

TX indicates transplantation; BP, blood pressure; CYA, cyclosporin A; MMF, mycophenolate mofetil; FK506, tacrolimus; NS, not significant; and NA, not applicable.

\*Year average of mean BP.

†Patients were receiving one of the following antihypertensive drugs or a combination of these: calcium channel blockers, clonidine, diuretics, ACE inhibitors, and  $\beta$ -blockers.

Review Board of The Methodist Hospital and Baylor College of Medicine. The cohort comprised patients who underwent cardiac and lung transplantation at The Methodist Hospital/Baylor College of Medicine Multi-Organ Transplant Center. Heart transplant recipients were followed up with sequential and clinically indicated endomyocardial biopsies. Serial myocardial biopsy samples were obtained from 9 patients within the first year of transplant. Detailed clinical characteristics of the population studied are shown in the Table. In addition, all heart transplant recipients tested positive for cytomegalovirus at the time of transplantation and received ganciclovir prophylaxis. For comparative analysis of cardiac TNF- $\alpha$  concentrations in transplant myocardium, we included 4 patients with depressed ejection fraction after transplantation who had no evidence of coronary artery disease by coronary angiography and no evidence of infection or rejection. Two patients underwent retransplantation, and after workup for reversible causes, 2 primary transplants were defined as nonspecific graft failures.

**Measurements of Left Ventricular Mass**

Assessment of left ventricular (LV) mass was performed by 2D echocardiography by application of the cube formula to the end-diastolic dimension and the septal and posterior wall thicknesses of the LV in the parasternal long-axis view.<sup>12</sup> For heart transplant recipients, baseline 2D echocardiograms of the donor heart were studied before explantation. For lung transplant recipients, the baseline echocardiogram was studied at the time of pretransplant evaluation. The follow-up study was conducted 12 months after transplantation.

**Source of Human Myocardium**

Control myocardium was obtained from donor hearts that were not used for cardiac transplantation. For titration experiments, normal myocardium was obtained from the pathology archives from autopsy specimens. Transplant myocardium was obtained from heart transplant recipients followed up by the transplant program at The Methodist Hospital. For analysis of myocyte size, total collagen content, and collagen I, collagen III, and serial TNF- $\alpha$  levels, we used samples from 9 consecutive patients from whom we had a complete set of histological samples. Heart transplantation in this population was performed between January 1997 and January 1998.

**Myocardial Biopsies**

All analysis was performed in random order by observers who were blinded to the sample source and date of acquisition. Myocardial biopsy samples (0.5 to 1 mg; 4 per patient) were obtained with a biotome under fluoroscopic guidance from the right ventricular side of the septum. Myocardial tissue samples were immediately immersed in 2% paraformaldehyde for 45 minutes and dehydrated in increasing concentrations of alcohols, then cleared through xylene and subsequently embedded in paraffin. Five-micrometer-thick sections were taken, then rehydrated through water. Sections were stained, then examined with an Olympus AX70 fluorescence microscope.

**Myocyte Size**

Hematoxylin-cosin staining was used to measure myocyte size. A point-to-point perpendicular line was drawn across the cross-sectional area of the myocytes at the level of the nucleus, and the computer imaging software then measured this diameter length. Transverse or oblique-sectioned myocytes were excluded. Thirty myocytes per slide were measured from each tissue specimen, and results were expressed as mean and SEM.

**Collagen Content**

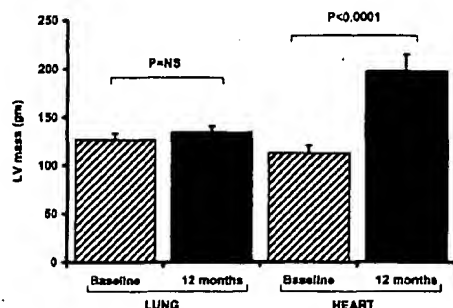
Myocardial tissue sections were stained with picosirius red as described previously.<sup>13-15</sup> Total collagen content was the sum of all areas stained within the slide, including interstitial, perivascular, and microscopic scars. Collagen I and III content was determined by staining myocardial tissue sections with a polyclonal rabbit anti-human collagen I or collagen III antibody (Accurate Chemical & Scientific Corp), respectively, at a concentration of 1/20.

**Myocardial TNF- $\alpha$  Levels**

Myocardial TNF- $\alpha$  content was measured with semiquantitative analysis of TNF- $\alpha$ -stained myocardial tissue. For TNF- $\alpha$  immunostaining, we used a polyclonal rabbit anti-TNF- $\alpha$  antibody (R&D Systems, Inc/Genzyme) at a 1/300 dilution. Staining was performed with a kit (Vector Laboratories, Inc) that used a peroxidase-conjugated avidin-biotin system and diaminobenzidine as a substrate. For preliminary experiments, myocardial samples were stained at varying concentrations of anti-TNF- $\alpha$  antibody ranging from a 1/10 to a 1/1000 dilution. Peak staining always occurred at a 1/300 dilution, and this concentration of antibody was used for all subsequent studies.

**Quantitative Analysis of Stained Areas**

Four microscopic fields were photographed per specimen slide with a Diagnostic Instrument Spot II color camera (Diagnostic Instrument, Inc) mounted on an AX70 fluorescence microscope (Olympus, Inc). All fields were digitized to a computer database and stored for analysis. Staining was analyzed with Image-Pro Plus 4.0 analysis software (Media Cybernetics) with color cube-based selection criteria for positive staining. Both intensity level (range) and area were analyzed according to the method of Matsuo et al.<sup>16</sup> Results in this article are based on area of positive staining within the color spectrum for diaminobenzidine for TNF- $\alpha$  and 3-amino-9-ethylcarbazole for collagen I and III at all intensities greater than those found in control antibody (IgG)-stained sections without correction for intensity. For total collagen and collagen I and III isoforms, all tissue specimens were obtained, processed, and analyzed in the same manner and results expressed as mean and SEM. Slides were analyzed by an investigator blinded both to the origin of the samples and to whether the slides originated from tissue obtained within the first week after transplantation or the twelfth month after transplant, according to our clinical operational procedure. However, because variation exists between the intensity of the staining from one experiment to the other, comparisons between cohorts were only performed within the same experiment. Although absolute values varied from experiment to experiment, the relative amount of immune-positive areas did not change.



**Figure 1.** Gain in LV mass after transplantation in heart and lung transplant recipients. LV mass was determined at baseline and at 12 months after transplantation in heart ( $n=10$ ) and lung ( $n=18$ ) recipients. Baseline measurements were obtained in lung transplant patients at time of transplant evaluation and for heart transplant recipients in donor heart before transplantation. Baseline LV mass was equal among heart and lung transplant patients. At 12 months, LV mass was significantly greater in heart transplant recipients ( $P<0.0001$ ).

### Statistical Analysis

Statistical analysis was performed by Mann-Whitney  $U$  tests to identify statistical significance between groups. All data in the text and figures are expressed as mean  $\pm$  SEM.

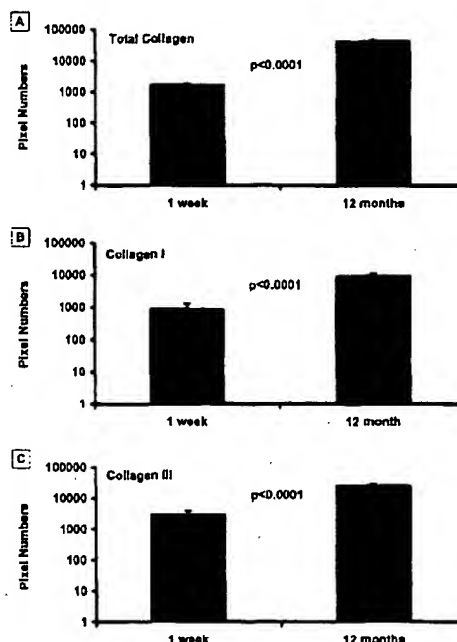
### Results

#### Systemic Hypertension Does Not Play a Primary Role in the Development of Cardiac Allograft Hypertrophy

To separate the effect of hypertension from that of the immune response against the graft in the development of hypertrophy, we compared the gain in LV mass that occurred in heart transplant recipients versus the gain in LV mass of patients who underwent lung transplantation within the first year. The demographics of the population studied are shown in the Table. Importantly, there were no significant differences with regard to degree of hypertension, immunosuppressive therapy, or age between the 2 groups; however, heart transplant recipients had a much greater gain in LV mass within the first year than lung transplant recipients (73% versus 7%,  $P<0.0001$ ; Figure 1).

#### Characterization of Hypertrophy After Cardiac Transplantation

Figure 2 shows the change in cardiac content of total collagen, collagen I, and collagen III that occurred during the first year after heart transplantation. No patient demonstrated a reduction in collagen content. The gain in total collagen was significant ( $P<0.0001$ ), as were the gains in collagen I ( $P<0.0001$ ) and collagen III ( $P<0.0001$ ) content. Figure 3 illustrates a representative immunostaining performed at baseline and at 12 months for total collagen, collagen I, and collagen III. The gain in size within the first year after transplantation is shown in Figure 4. A gain in myocyte size was observed in all patients studied. The mean gain compared with baseline was 47% ( $P<0.0001$ ; Figure 4A). Myocyte size increased progressively over time (Figure 4B).



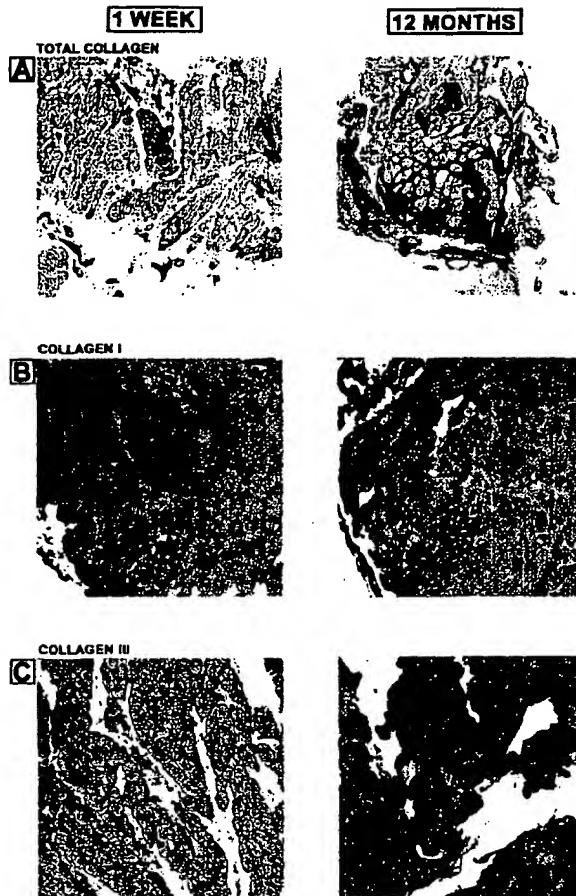
**Figure 2.** Change in collagen content after cardiac transplantation. Semiquantitative determination of total collagen (A), collagen I (B), and collagen III (C) content was determined at 1 week and at 12 months after cardiac transplantation ( $n=9$ ). Determination of collagen content is expressed in pixels as arbitrary units.

#### TNF- $\alpha$ Is Persistently Expressed in Cardiac Allografts

Figure 5A shows the mean cardiac TNF- $\alpha$  value for controls ( $n=2$ ) and for heart transplant recipients who manifested no evidence of cellular rejection ( $n=8$ ). There was a significant increase in TNF- $\alpha$  levels in transplant myocardium, as well as absent or very low immunodetectable TNF- $\alpha$ . A representative tissue section for control and transplant myocardium stained for TNF- $\alpha$  is shown in Figure 5B. As shown, there was significant TNF- $\alpha$  expression in transplant myocardium, whereas minimal or no stain was seen in control. To further characterize the expression of TNF- $\alpha$  over time, we determined intracardiac TNF- $\alpha$  levels in serial samples obtained from 9 patients over the first 12 months after transplantation. We found that TNF- $\alpha$  levels were higher within the first month after transplantation, and although they tended to decrease, they remained abnormally elevated throughout the first year (Figure 5C).

#### TNF- $\alpha$ Expression Is Not Associated With Rejection Scores or Systolic Dysfunction

To define whether TNF- $\alpha$  was produced in response to allograft rejection, we determined TNF- $\alpha$  levels in patients with various rejection scores as determined by the International Society for Heart and Lung Transplantation grading system.<sup>17</sup> Figure 6A shows that increased intracardiac TNF- $\alpha$  levels were found in patients with low and high rejection scores and that there was no correlation between the degree of rejection and the intracardiac concentration of TNF- $\alpha$ . Next, because TNF- $\alpha$  is capable of causing negative inotropic



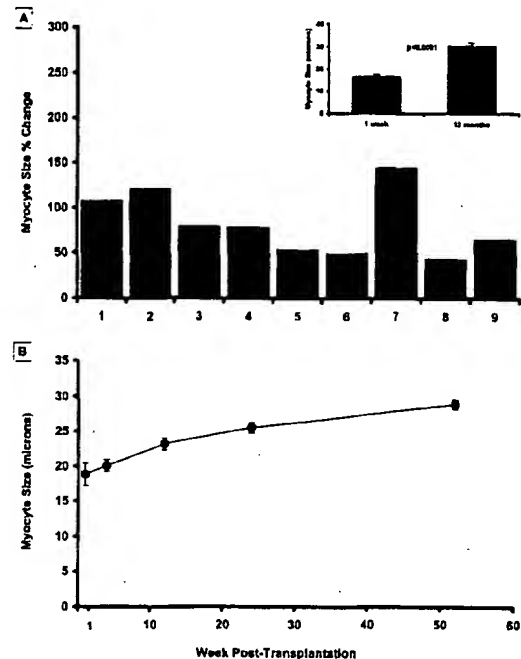
**Figure 3.** Representative immunostaining of various collagen isoforms after transplantation. Endomyocardial biopsy samples obtained within first week after transplantation (left) and at 12 months (right) were stained for total collagen (A), collagen I (B), and collagen III (C). (Original magnification  $\times 20$ ).

effects under acute experimental conditions, we determined whether myocardium chronically exposed to  $\text{TNF-}\alpha$  had abnormal systolic function. Figure 6B shows intracardiac  $\text{TNF-}\alpha$  levels in heart transplant recipients with normal ( $n=5$ ) and depressed systolic function ( $n=4$ ). There was no difference in intracardiac  $\text{TNF-}\alpha$  concentration between these 2 groups ( $P=\text{NS}$ ).

### Discussion

The major finding of the present study was the demonstration that cardiac allograft hypertrophy occurs independently of the development of systemic hypertension and in association with persistent expression of  $\text{TNF-}\alpha$ , a cytokine capable of producing hypertrophy and fibrosis.

The severity of the hypertrophic response, as well as its rapid appearance after transplantation, suggested that perhaps immune cardiac injury played a contributory role. To address this issue further, we needed a strategy by which we could separate the effect of hypertension from that of the immune injury in the development of cardiac allograft hypertrophy. We reasoned that if the development of posttransplant ac-

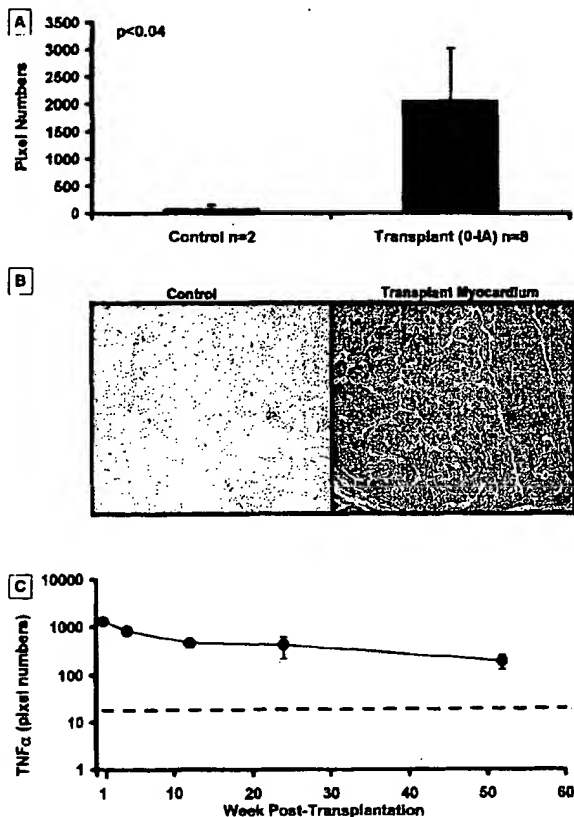


**Figure 4.** Change in myocyte size after transplantation. A, Myocyte size was determined at 1 week and at 12 months after transplantation. Data are expressed in micrometers for mean values of 9 patients as determined at 1 week and at 12 months. B, Change in myocyte size over time in population studied. Data are mean  $\pm$  SEM.

quired hypertension was the major contributor to the development of LV hypertrophy, then lung transplant recipients, who develop similar rates of hypertension, would also develop LV hypertrophy. However, when we compared the change in LV mass in lung and heart transplant recipients, we found a much greater gain in LV mass in the heart transplant group. This finding indicated that transplant-acquired hypertension was not sufficient to explain the development of cardiac allograft hypertrophy.

Next, we characterized the histological changes that occur in cardiac allografts after transplantation in collagen content and myocyte size. We found that there was a significant increase in the amount of total collagen content, as well as in collagen I and collagen III. In association with the changes observed in collagen content, we found a significant increase in myocyte size after transplantation. Our findings are consistent with previous observations<sup>18</sup> that demonstrated that cardiac allograft hypertrophy was the result of an increase in cell size and extracellular matrix deposition of collagen, leading to increased fibrosis.

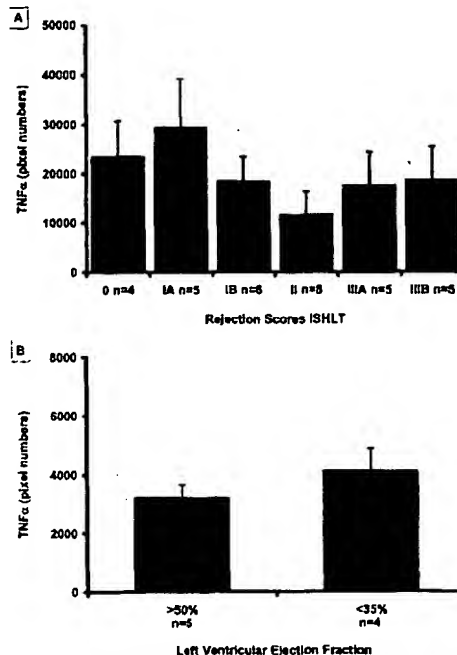
Recent experimental observations indicate that calcineurin-mediated responses are important in the development of cardiac hypertrophy; indeed, in various animal models, calcineurin inhibition attenuates the development of cardiac hypertrophy.<sup>19-21</sup> The findings of the present study indicate that despite therapeutic doses of either cyclosporine or tacrolimus, cardiac allograft hypertrophy occurs. Whether the role of calcineurin in this setting is not important or the doses used clinically are not sufficient to attenuate the process of



**Figure 5.** Expression of TNF- $\alpha$  in cardiac allografts. A, Tissue sections of control (n=2) and transplant myocardium (n=8) were stained for TNF- $\alpha$ . Semiquantitative analysis of stained area was performed as detailed in Methods. As shown, control myocardium does not contain TNF- $\alpha$ , whereas transplant myocardium expressed high levels of intracardiac TNF- $\alpha$ . 0-1A indicates rejection score 0 or 1A. B, Representative tissue sections of control (left) and transplant myocardium (right) from patient with low rejection score (1A) were stained for TNF- $\alpha$ . Control myocardium did not stain for TNF- $\alpha$ , whereas there was abundant staining in transplant myocardium. Positive immunostaining was present in myocytes. (Original magnification  $\times 20$ ). C, Intracardiac TNF- $\alpha$  levels were determined by semiquantitative immunohistochemistry in serial samples obtained from 9 patients over first 12 months after transplantation.

hypertrophy is not known. Clearly, cardiac allograft hypertrophy does not appear to be altered by the presence of therapeutic doses of calcineurin inhibitors.

Because hypertension was not the primary force for the development of hypertrophy, and because the heart is the target of immune responses directed to cause rejection in heart transplant recipients, it was logical to assume that either the cellular response or a byproduct of the immune response against the heart played a contributory role in cardiac allograft hypertrophy. To evaluate this concept further, we characterized the expression of TNF- $\alpha$ , a cytokine present in transplant myocardium<sup>10</sup> and capable of producing hypertrophy and cardiomyopathy.<sup>7,8,20</sup> Indeed, in the present study, we found that TNF- $\alpha$  was persistently expressed in serial myocardial samples of heart transplant recipients and that its expression was not determined by the severity of rejection



**Figure 6.** Relationship of cardiac TNF- $\alpha$  to rejection scores and systolic function. A, Random endomyocardial biopsy samples representative of various International Society for Heart and Lung Transplantation (ISHLT) scores were used to measure TNF- $\alpha$ . There was no correlation between TNF- $\alpha$  level and histological score of rejection ( $P=NS$ ). B, Cardiac TNF- $\alpha$  levels were determined in each of 6 endomyocardial biopsy samples obtained at 1 week and 1, 2, 3, 4, 5, and 6 months after transplantation. Cardiac TNF- $\alpha$  represents sum of individual values. There was no difference in cardiac TNF- $\alpha$  content among patients with preserved or decreased LV function.

scores. Furthermore, we found that in patients with high myocardial TNF- $\alpha$  content, LV systolic function was preserved.

The observation that TNF- $\alpha$  is persistently expressed in human myocardium in association with hypertrophy and preserved LV function is consistent with observations in transgenic mice with cardiac-restricted TNF- $\alpha$  overexpression that demonstrate significant hypertrophy and maintained systolic function before the development of a dilated phenotype.<sup>7,8</sup> In the present studies, we determined intracardiac and not peripheral TNF- $\alpha$  levels because it has been demonstrated that there is no correlation between serum and cardiac TNF- $\alpha$  levels.<sup>3</sup> Also, the biological effects of TNF- $\alpha$  correlate with intracardiac and not with peripheral TNF- $\alpha$ .<sup>4</sup> In this regard, the data presented herein reflect cardiac TNF- $\alpha$  concentrations that can potentially affect cardiac function.

Although the present results do not provide direct evidence that TNF- $\alpha$  is responsible for the development of fibrosis or hypertrophy, a large number of experimental reports suggest that such may be the case. First, studies performed in isolated cardiac myocytes demonstrated that TNF- $\alpha$  increased protein synthesis.<sup>22</sup> Second, in the intact heart, TNF- $\alpha$  induced expression of angiotensin II, a known mediator of fibrosis and cardiac growth.<sup>23</sup> And finally, there are at least 2 independent lines of transgenic mice with cardiac-restricted overexpres-

sion of TNF- $\alpha$  that are characterized by rapid development of hypertrophy and fibrosis.<sup>7,8</sup>

In summary, the findings of the present study demonstrate that transplant myocardium is not "normal." It develops hypertrophy in an accelerated manner despite therapeutic interventions and expresses increased levels of TNF- $\alpha$ , a cytokine capable of stimulating cardiac growth. We suggest that TNF- $\alpha$  may be a mediator of hypertrophy and fibrosis in cardiac allografts, and we provide the experimental basis to conduct clinical trials aimed to block the biological effects of TNF- $\alpha$  in heart transplant recipients.

### Study Limitations

The number of patients in this study was small, and the amount of endomyocardial tissue obtained during biopsies did not permit protein analysis by multiple techniques. For these studies, we chose to perform semiquantitative analysis based on immunohistochemistry, because this would permit the measurement of multiple proteins in the same tissue. The techniques used in these experiments, however, have been validated by studies from our laboratory and others.<sup>7,13-15,23</sup>

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